# XCoe2, a transcription factor of the Col/Olf-1/EBF family involved in the specification of primary neurons in *Xenopus*

Laurence Dubois*, Laure Bally-Cuif†§, Michèle Crozatier*, Jacques Moreau‡, Laurent Paquereau* and Alain Vincent*

**Background**: Primary neurogenesis in *Xenopus* is a model for studying the control of neural cell fate decisions. The specification of primary neurons appears to be driven by transcription factors containing a basic region and a helix–loop–helix (HLH) motif: expression of *Xenopus neurogenin-related-1* (*X-ngnr-1*) defines the three prospective domains of primary neurogenesis, and expression of *XNeuroD* coincides with neuronal differentiation. The transition between neuronal competence and stable commitment to a neuronal fate remains poorly characterised, however.

**Results**: *Drosophila* Collier and rodent early B-cell factor/olfactory-1 define a family of HLH transcription factors containing a previously unknown type of DNA-binding domain. We isolated an orthologous gene from *Xenopus*, *Xcoe2*, which is expressed in precursors of primary neurons. *Xcoe2* is transcribed after *X-ngnr-1* and before *XNeuroD*. Overexpression of a dominant-negative mutant of XCoe2 prevented neuronal differentiation. Conversely, overexpressed wild-type *Xcoe2* could promote ectopic differentiation of neurons, in both the neural plate and the epidermis. In contrast to studies with *X-ngnr-1* or *XNeuroD*, the supernumerary neurons induced by *Xcoe2* appeared in a 'salt-and-pepper' pattern, resulting from the activation of *X-Delta1* expression and feedback regulation by lateral inhibition.

**Conclusions**: XCoe2 may play a pivotal role in the transcriptional cascade that specifies primary neurons in *Xenopus* embryos: by maintaining Delta–Notch signalling, XCoe2 stabilises the higher neural potential of selected progenitor cells that express *X-ngnr-1*, ensuring the transition between neural competence and irreversible commitment to a neural fate; and it promotes neuronal differentiation by activating *XNeuroD* expression, directly or indirectly.

Addresses: *Centre de Biologie du Développement, UMR 5547, CNRS/Université Paul Sabatier, 118 route de Narbonne, 31062 Toulouse Cedex, France. †Department of Molecular Biology, Princeton University, Princeton, New Jersey 08540, USA. ‡Institut Jacques Monod, CNRS et Université Paris 7, 2 Place Jussieu, 75251 Paris cedex 05, France.

Present address: §CNRS URA1414 Equipe Atipe, ENS, 46 rue d'Ulm, 75005 Paris, France.

Correspondence: Alain Vincent
E-mail: vincent@cict.fr

Received: 4 November 1997
Revised: 19 December 1997
Accepted: 19 December 1997

Published: 27 January 1998

Current Biology 1998, 8:199–209
http://biomednet.com/elecref/0960982200800199



## Background

Neural development in vertebrates starts during gastrulation with the demarkation of the neural plate, which is initially a field of undifferentiated, mitotically active cells. In anamniotic vertebrates such as *Xenopus* or the zebrafish, a first wave of cells withdraws from the mitotic cycle early during embryogenesis to generate a simple pattern of primary neurons that control early larval behaviour [1]. This early and precisely scheduled pattern makes primary neurogenesis in *Xenopus* an attractive model for the study of molecular and cellular mechanisms involved in neural cell fate decisions. *Xenopus* studies have shown that despite the gross anatomical and developmental differences between the vertebrate and *Drosophila* central nervous systems (CNSs), remarkably similar sets of genes and mechanisms are involved in neurogenesis in the two species [2,3].

The genetic control of neuroblast formation has been studied in considerable detail in *Drosophila*. Expression of a group of regulatory genes, the proneural genes, which encode transcription factors containing a basic region associated with a helix–loop–helix dimerisation motif (bHLH factors), is believed to render groups of cells competent to form neuronal progenitors. The selection of neuronal progenitors from each group of competent cells involves lateral inhibition, a cell–cell signalling mechanism by which cells committed to differentiate force neighbouring cells to remain uncommitted [2,4]. In this process, the inhibitory signal Delta emitted by the nascent neural cells activates its transmembrane receptor Notch on adjacent cells [5]. Whereas the early action of proneural genes promotes the expression of Delta, expression of the proneural genes is down-regulated by activated Notch [6,7]. It has been proposed that this feedback mechanism magnifies the small differences between adjacent cells in their levels of proneural gene expression; cells initially expressing higher levels of proneural activity than their neighbours become less sensitive to lateral inhibition and are consequently committed to a neural fate [8]. The nature of the events that stabilise neural fate and commit

a cell irreversibly to neuronal differentiation have not yet been completely elucidated, however.

In *Xenopus*, the neuronal progenitors that give rise to the primary (larval) nervous system form in the neural plate in three longitudinal domains — medial, intermediate and lateral. The generation of these neurons is regulated by lateral inhibition mediated by Delta–Notch signalling in the same way as in *Drosophila*. Two recently identified bHLH transcription factors, *Xenopus* neurogenin-related-1 (X-Ngnr-1) and XNeuroD, are expressed in the progenitors of primary neurons [9,10]. Expression of the *X-ngnr-1* gene defines the three prospective domains of primary neurogenesis, and *X-ngnr-1* has been proposed to act as a neuronal determination gene, perhaps analogous to the *Drosophila* proneural genes, whereas *XNeuroD* appears to act later, in the neuronal differentiation of committed cells. The temporal separation between *X-ngnr-1* expression and the activation of *XNeuroD* suggested that intermediate factors in the cascade of transcriptional activation specifying the primary neuron progenitors were yet to be identified [10].

We have cloned the *Xenopus* gene *Xcoe2*, which encodes a different transcription factor involved in the neurogenic cascade, by means of its homology to the rodent early B-cell factor/olfactory-1 (EBF/Olf-1) and *Drosophila* Collier (Col) transcription factors. The *col* gene was isolated as a regulator of *Drosophila* head patterning [11], and EBF/Olf-1 was characterised independently both as a B-cell-specific factor (EBF [12]) and as an olfactory-neuron-specific factor (Olf-1 [13]). These proteins define a family of transcription factors with a type of DNA-binding domain associated with a HLH motif [11,14] that had not been described previously. Two additional mouse genes of the same family have been identified recently: EBF-2/OE3/Mmot1 and EBF-3/OE2 [15–17]. Although slightly different results were reported concerning the respective timing and specificity of expression of each mouse EBF/OE gene, all three genes are transiently expressed in a subset of precursor cells in the embryonic CNS and the peripheral nervous system, concomitantly with the initiation of overt differentiation within the nervous system [15–17]. We have turned to the *Xenopus* embryo in order to investigate the early embryonic functions of the Col/Olf-1/EBF family of transcription factors. We show here that XCoe2, the homologue of EBF-2, is an essential component of primary neurogenesis in *Xenopus*, perhaps acting to connect X-Ngnr-1 and XNeuroD functions.

## Results

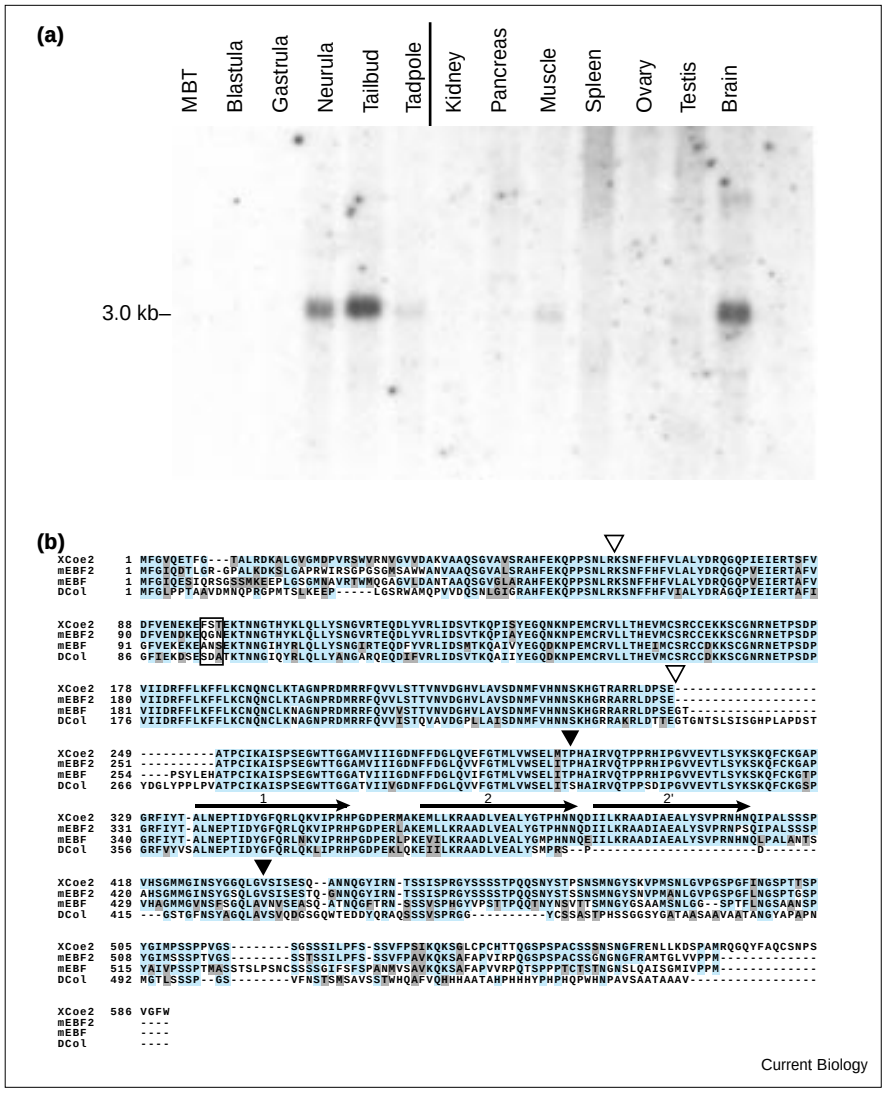
### Identification and embryonic expression of a *Xenopus* gene related to *Drosophila collier* and mouse *EBF*

To isolate *Xenopus* embryonic cDNAs related to the *Drosophila collier* and mouse *EBF* genes, we performed reverse transcriptase PCR using cDNAs from stage 25 embryos and degenerate oligonucleotide primers derived from conserved sequences flanking the DNA-binding domain of Col and EBF/Olf-1. (The same strategy, amplifying related sequences in the mouse, led to the isolation of EBF-2 and EBF-3 [15].) DNA fragments of the expected size (560 bp) were amplified and cloned in bacteria. Analysis of 11 independent clones revealed coding sequences highly related to the Col/EBF DNA-binding domain although differing from each other at a few positions specific to each clone. Three of the eleven clones were identical, and the cDNA thus defined was used to probe a northern blot containing RNA from *Xenopus* embryos at different developmental stages. A single transcript, about 3 kb in length, was detected in neurula-stage embryos (data not shown). The same fragment was therefore used to screen a cDNA library from stage 17 *Xenopus* embryos [18]. Of $10^6$ phages tested, six positive clones were isolated, differing only by the length of their 5′ ends.

Comparison between the largest cDNA obtained and the products of rapid amplification of cDNA ends (RACE) indicated that it was full length. Hybridisation of this cDNA clone to a developmental northern blot confirmed the zygotic expression of a 3 kb transcript, with expression starting at the neural plate stage and with a peak of transcript accumulation at the tailbud stage. A transcript of the same size was also detected in a subset of adult tissues: at high levels in the brain and low levels in the somatic muscles, testis, and possibly the spleen (Figure 1a and data not shown). Nucleotide sequencing revealed an open reading frame of 1767 nucleotides, predictive of a 589 amino-acid protein (Figure 1b) highly related to Col and EBF/Olf-1. We named this gene *Xcoe2* (*Xenopus* member of the Col/Olf-1/EBF family of transcription factors). Sequence alignment between XCoe2, Col, EBF, EBF-2 and EBF-3 (data not shown) confirmed the presence of two particularly well-conserved regions corresponding to the DNA-binding domain and the dimerisation domain, which includes an HLH motif (Figure 1b) [11]. XCoe2 appears, however, more closely related to EBF-2 than to either EBF or EBF-3, hence the suffix 2.

Comparison of the Col, EBF and XCoe2 amino-acid sequences with other protein sequences in the current databases revealed an extensive similarity with a predicted *Caenorhabditis elegans* open reading frame, which probably corresponds to the protein product of the *unc-3* gene, originally identified in the genetic screen of S. Brenner [19] (T. Starrich, personal communication). A comparison of the predicted DNA-binding domains of Col, EBF and XCoe2 shows a small stretch of two or three amino-acid residues (boxed in Figure 1b), specific to each protein, that might be considered a gene-specific ‘signature’. The same signature was present in all 11 PCR products amplified from stage 25 *Xenopus* embryos, suggesting that during primary neurogenesis in *Xenopus Xcoe2* is the most highly, if not the only, expressed *coe* gene.

**Figure 1**



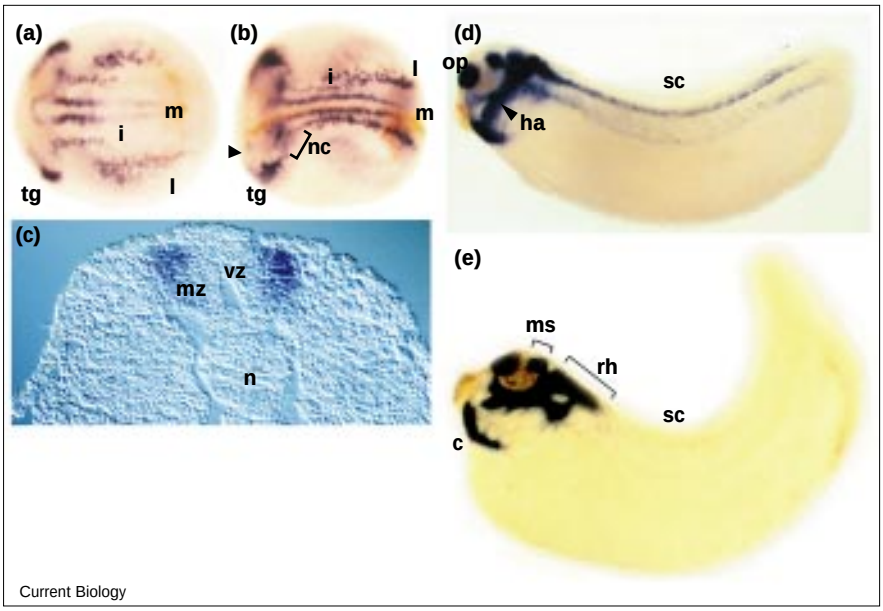

(a) A profile of *Xcoe2* transcript accumulation during development and in adult tissues. A northern blot containing RNA from different developmental stages was probed with labelled 2.2 kb *Xcoe2* cDNA. The developmental stage of each embryonic RNA is indicated. No hybridisation signal was detected with RNA from oocytes at any stage of oogenesis (data not shown). A 3 kb *Xcoe2* transcript is present in embryos starting at the neural plate stage. A transcript of the same size is detected in specific adult tissues and is most abundant in muscle, testis and brain. Each lane contains 10 μg total RNA. We used ribosomal RNA, visualised by ethidium bromide staining, as an internal standard for quantification of deposited RNA (data not shown). MBT, mid-blastula transition. (b) Amino-acid sequence alignment of *Xenopus* XCoe2 with other members of the EBF/Col family: mouse EBF and EBF2 and *Drosophila* Col. Identical and similar amino acids are indicated by blue and grey shading, respectively. The DNA-binding domain, framed by open arrowheads, extends from EBF/Olf-1 amino acids 50 to 251 and includes a zinc-binding motif (amino acids 151–177). The second highly conserved domain is framed by black arrowheads. The horizontal arrows indicate the positions of helix 1 and helix 2 of a predicted HLH motif which is conserved in all four proteins; a duplicated helix 2 (labelled 2′) is present in the vertebrate proteins. A stretch of two or three amino acids specific to each protein is boxed. Dashes represent spaces to optimise the alignment.

### *Xcoe2* is an early marker of primary neurogenesis

To examine the role of *Xcoe2* in *Xenopus* embryos, we first analysed its early embryonic expression by whole-mount *in situ* hybridisation. *Xcoe2* transcripts were first detected at the open neural-plate stage (stage 12), in a pattern anticipating that of the neuron-specific *N-tubulin* gene (Figure 2a; see also Figure 3). *N-tubulin* expression was previously shown to mark the formation of three groups of primary neurons, arranged in a radially symmetrical pattern on either side of the dorsal midline, in the posterior neural plate. The medial (future ventral), intermediate and lateral (future dorsal) groups of neurons will later differentiate into motorneurons, interneurons and sensory neurons, respectively. Expression of *Xcoe2* completely overlapped that of *N-tubulin* at stage 13, and this overlap included expression in the trigeminal placodes (Figure 2a). *Xcoe2* is therefore a new early marker of primary neurogenesis. At around stage 17, *Xcoe2* began to be expressed in groups of anterior cells associated with the olfactory placodes, and in prospective anterior neural crest cells (Figure 2b). When the neural tube closed, *Xcoe2* expression in the spinal cord was restricted to the lateral zone, being absent from the mitotically active ventricular zone (Figure 2c), as has already been observed for mouse EBF-2 [15]. From stage 28, this expression faded gradually, in an antero-posterior gradient, to become undetectable at stage 32 (compare Figure 2d and 2e). These observations show that *Xcoe2* is expressed only transiently in post-mitotic neurons.

Between stages 20 and 28, neural crest cells contributing to the mandibular, hyoid and third branchial arches express *Xcoe2* (Figure 2d,e). These cells, which migrate ventrally between the ectoderm and the dorsal mesoderm,

**Figure 2**




*Xcoe2* mRNA expression during *Xenopus* development, revealed by whole-mount *in situ* hybridisation. Embryos at stages (a) 14, (b) 17, (c,d) 28 and (e) 32 are shown. Anterior is to the left; (a,b) dorsal view; (d,e) lateral view. The three stripes of primary neuron precursors, medial (m), intermediate (i) and lateral (l) and the trigeminal ganglia (tg), are indicated. In (b), the positions of nascent olfactory placodes (op; arrowhead) and anterior neural crest cells (nc) are also indicated. (c) Transverse section at the level of the spinal cord (sc). *Xcoe2* expression is restricted to differentiating neurons in the marginal zone. This expression is still observed at stage 28 (d) but no longer at stage 32 (e). Other abbreviations: c, cartilage; ha, hyoid arch; ms, mesencephalon; mz, marginal zone; n, notocord; rh, rhombencephalon; vz, ventricular zone.

may participate in the formation of the cartilaginous skeleton [20]. Other *Xcoe2*-expressing neural crest cells will contribute to the cranial ganglia. At stage 32, *Xcoe2* expression remained strong in specific groups of cells in the mesencephalon and the rhombencephalon where neuronal differentiation was still proceeding, and in the olfactory placodes and cranial ganglia (Figure 2e). *Xcoe2* expression in early embryos was not restricted to neurectodermal cells, but also occurred transiently in cells located at the border between somites and lateral plates before these two mesodermal derivatives become separate (Figure 2d and data not shown).

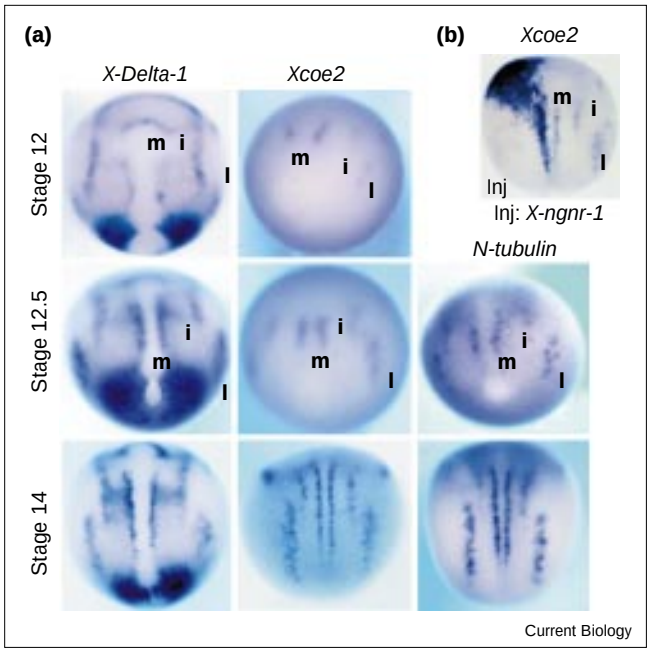
### *Xcoe2* expression follows *X-ngnr-1* and *X-Dl1* but precedes *N-tubulin* and *XNeuroD*

On the basis of its early expression in the neural plate and its regulation by lateral inhibition, *X-ngnr-1* has been proposed to be a proneuronal-like gene, encoding a protein that functions, at least in part, to activate *X-Delta-1* (*X-Dl1*) and neural differentiation genes such as *N-tubulin* and *XNeuroD* [10]. In order to investigate further the possible function of *Xcoe2* during primary neurogenesis, we compared in detail its temporal pattern of expression relative to the expression of these other genes. The earliest expression of *Xcoe2* occurs at stage 12, after *X-Dl1* and *X-ngnr-1* expression has become restricted to the prospective neuronal progenitors by lateral inhibition (Figure 3a) [10,21]. *Xcoe2* expression anticipates the pattern of all primary neurons as marked by *N-tubulin* expression at stage 12.5. It also anticipates the expression of *XNeuroD*, which is not detected prior to stage 13.5, and then only in a subset of cells expressing *Xcoe2* and *X-ngnr-1* ([9,10] and data not shown). The sequential order of transcription of the known HLH transcription factors involved in *Xenopus* primary neurogenesis therefore starts with *X-ngnr-1*, followed by *Xcoe2* and then, significantly later, *XNeuroD*. In order to determine whether *Xcoe2* expression was controlled by *X-ngnr-1*, we injected *X-ngnr-1* mRNA into one blastomere of embryos at the two-cell stage and looked at *Xcoe2* mRNA at developmental stage 14 by *in situ* hybridisation. A *lacZ* mRNA marker was co-injected and the distribution of β galactosidase activity was used to assess the distribution of the injected mRNAs. All 26 injected embryos examined showed ectopic *Xcoe2* mRNA on the injected side, in both the neural plate and the epidermis, indicating that *X-ngnr-1* ectopically activates *Xcoe2* transcription (Figure 3b). Together, the temporal and spatial expression of *Xcoe2* and its activation by *X-ngnr-1* suggested that *Xcoe2* acts at an early stage in the neurogenic cascade, upstream of neuronal differentiation genes such as *XNeuroD*.

### Decreasing XCoe2 activity prevents neuronal specification

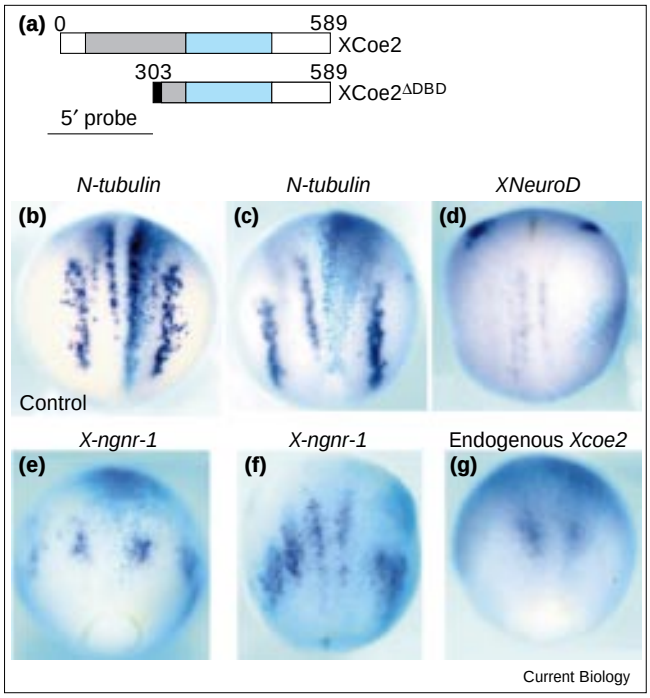
To determine whether *Xcoe2* is required for the specification of primary neurons, we attempted to decrease XCoe2 activity in embryos by overexpressing a dominant-negative mutant form of the protein. The design of this mutant protein, XCoe2$^{\Delta DBD}$, was based on the functional dissection of the EBF/Olf-1 protein *in vitro* [12,14] and on the high structural conservation of the dimerisation region and DNA-binding domain in all members of the Coe family (Figure 1b). XCoe2$^{\Delta DBD}$ has a truncation that removes the DNA-binding domain but leaves the dimerisation domain intact (Figure 4a). We hypothesised that

**Figure 3**




*X-Dl1*, *Xcoe2* and *N-tubulin* are activated sequentially in prospective primary neurons. (a) Expression of *X-Dl1*, *Xcoe2* and *N-tubulin* during early *Xenopus* embryogenesis was analysed by whole-mount *in situ* hybridisation. Probes and stages of embryonic development are indicated. All embryos are shown in a dorsal view, with the anterior to the top. The three stripes of primary neurons, medial (m), intermediate (i) and lateral (l), are indicated. (b) One cell (inj) of an embryo at the two-cell stage was injected with synthetic *X-ngnr-1* (10 pg) and *lacZ* (50 pg) mRNAs. *In situ* hybridisation to a stage 14 embryo with a probe to *Xcoe2* shows the ectopic activation of *Xcoe2* by X-Ngnr-1.

**Figure 4**




*Xcoe2* is required for the differentiation of primary neurons in the *Xenopus* embryo. (a) The epitope-tagged deletion mutant *Xcoe2*$^{\Delta DBD}$ was constructed by removing the region encoding the DNA-binding domain (grey) almost entirely and leaving the homodimerisation domain (blue) intact [14]. The c-Myc epitope tag of Xcoe2$^{\Delta DBD}$ is indicated (black) at the amino terminus. Embryos were microinjected with the following mRNAs: (b) *lacZ* (500 pg) or (c–g) *lac Z* (50 pg) and *Xcoe2*$^{\Delta DBD}$ (500 pg), followed by *in situ* hybridisation of embryos at stage 13.5–14 with either (b,c) *N-tubulin* probe, or (d) *X-NeuroD* probe; embryos at (e) stage 11.5 and (f) stage 13 with *X-ngnr-1* probe; and (g) embryos at stage 12 with a 5′ *Xcoe2* probe, indicated in (a), that does not hybridise to *Xcoe2*$^{\Delta DBD}$ mRNA. All embryos are shown in a dorsal view with the injected side on the right.

overexpressing this truncated protein would titrate the native XCoe2 and prevent its normal function via the formation of XCoe2–XCoe2$^{\Delta DBD}$ heterodimers that are not able to bind DNA properly [12]. *Xcoe2*$^{\Delta DBD}$ and *lacZ* mRNAs were injected into embryos at the four-cell stage. As a result, cells expressing *N-tubulin* or *X-NeuroD* were absent or drastically reduced in number in the regions of the embryo expressing injected mRNA, as defined by staining for β-galactosidase activity (compare Figure 4b and c,d; 74% ($n$ = 65) and 72% ($n$ = 36) of embryos have reduced numbers of cells expressing *N-tubulin* and *XNeuroD*, respectively). This effect was completely reversed when mRNA encoding full-length XCoe2 was co-injected with *Xcoe2*$^{\Delta DBD}$ mRNA (data not shown), indicating that XCoe2$^{\Delta DBD}$ specifically antagonises XCoe2 activity. In order to determine at which stage XCoe2$^{\Delta DBD}$ blocked the neuronal determination process, we examined the patterns of expression of *X-ngnr-1* at stages 11.5 and 14, and activation of endogenous *Xcoe2* at stage 12, using the 5′-specific probe defined in Figure 4a. Normal patterns of *X-ngnr-1* and *Xcoe2* expression were observed at stage 11.5–12, (Figure 4e,g; 100% of embryos, $n$ = 26, expressed *X-ngnr-1* in a normal pattern, 84%, $n$ = 49, expressed *Xcoe2* in a normal pattern), indicating that XCoe2$^{\Delta DBD}$ blocks the neuronal specification process only after the onset of endogenous *Xcoe2* expression. We noted a significant reduction of *X-ngnr-1* expression as early as stage 12.5 on the injected side in 65% of embryos ($n$ = 40), however, suggesting that XCoe2 activity is required to maintain *X-ngnr-1* transcription in selected progenitor cells (Figure 4f). When embryos injected with *Xcoe2*$^{\Delta DBD}$ mRNA reached the tadpole stage, most did not show a proper escape response, being unable to contract on one side, a phenotype suggestive of unilateral innervation defects. Given also the sequence of expression *X-ngnr-1*, *Xcoe2* and *XNeuroD*, the block of neuronal differentiation resulting from overexpression of XCoe2$^{\Delta DBD}$ suggests that XCoe2 is essential for the specification of primary neurons in the *Xenopus* embryo, acting downstream of *X-ngnr-1* and upstream of *XNeuroD*. It may act

partly through a positive-feedback regulation of *X-ngnr-1* expression in selected progenitor cells.

### Ectopic *Xcoe2* induces ectopic neurogenesis in only scattered cells of both the neural plate and the epidermis

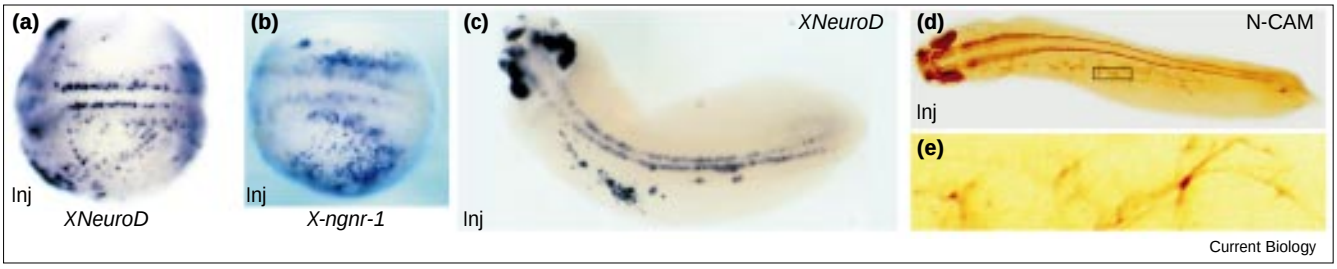
The results described above suggest that XCoe2 is necessary for primary neurogenesis, so we next asked whether XCoe2 is sufficient to induce ectopic neurogenesis. *Xcoe2* was overexpressed in *Xenopus* embryos by injecting *Xcoe2* mRNA (together with *lacZ* mRNA) at the two-cell or four-cell stage. The pattern of neurogenesis in *Xcoe2*-injected embryos was examined at various intervals by *in situ* hybridisation with probes for *XNeuroD* and *N-tubulin*. Overexpression of *Xcoe2* mRNA caused ectopic neurogenesis both within the neural plate and in regions of presumptive epidermis. At stage 15–16, scattered cells on the injected side appeared to express *XNeuroD* (Figure 5a,c; 65% of embryos, $n = 48$) or *N-tubulin* (71% of embryos, $n = 70$, data not shown) in the neural plate and epidermis, whereas the three stripes of primary neurogenesis were normal on the uninjected side (Figure 5a). Because $Xcoe2^{\Delta DBD}$ injection experiments indicated a feedback regulation of *X-ngnr-1* transcription by *Xcoe2* after stage 12, we examined *X-ngnr-1* expression in *Xcoe2*-injected embryos. Figure 5b shows that *X-ngnr-1* is ectopically activated in response to *Xcoe2* RNA overexpression and, as observed with *XNeuroD*, in a scattered cell pattern (86% of embryos, $n = 36$). At the tailbud stage (stage 25), cells expressing *N-tubulin* or *XNeuroD* ectopically were still observed within the epidermis (Figure 5c and data not shown). We verified that some of these cells differentiated into neurons by staining stage 30 embryos with antibodies directed against the neural cell adhesion molecule (N-CAM), a general marker of neural tissue that remains expressed during terminal neuronal differentiation (Figure 5d,e). Differentiated neurons with axonal projections were observed in the epidermis on the injected side of the embryo, confirming that *Xcoe2* overexpression is sufficient to promote neuronal fate in ectoderm that is normally non-neurogenic.

### The pattern of *Xcoe2*-induced ectopic neurons results from the ectopic activation of *X-Dl1* and lateral inhibition

Whereas in embryos injected with either *X-ngnr-1* mRNA or *X-NeuroD* nearly all cells of the neural plate on the injected side expressed *N-tubulin* [10], in embryos injected with *Xcoe2* mRNA, cells expressing *X-ngnr-1*, *N-tubulin* or *XNeuroD* always appeared scattered (Figure 5). This salt-and-pepper pattern suggested that *Xcoe2* neural function is regulated by lateral inhibition. As *X-Dl1* is not expressed in the epidermis (Figure 3a) [21], we first tested whether *Xcoe2* activates *X-Dl1* transcription. *In situ* hybridisation of embryos injected with *Xcoe2* mRNA showed that *X-Dl1* was ectopically expressed in the epidermis by stage 12.5 in 65% of embryos ($n = 109$). In contrast to *X-ngnr-1*, *XNeuroD* or *N-tubulin* (Figure 5), *X-Dl1* appeared to be ectopically activated more or less uniformly in all cells in the region of injection (Figure 6a). The ectopic *X-Dl1* transcripts became redistributed into patches of clustered cells during the developmental period between stages 14 and 17 (Figure 6b).

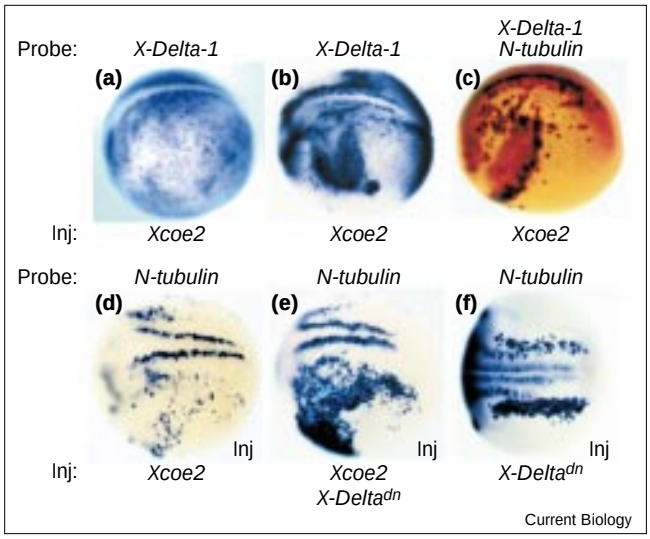
Although we have not investigated this patching in more detail, we noted that, during the same period, cells expressing ectopic *N-tubulin* or *X-NeuroD* (Figure 5c and data not shown) also redistributed into small patches (Figure 6c). Furthermore, *in situ* hybridisation with a combination of *X-Dl1* and *N-tubulin* probes showed that the *N-tubulin*-positive cells were mostly located within the patches of *X-Dl1*-expressing cells, but only at the border with cells that did not express *X-Dl1* (Figure 6c). This was consistent with the observation that production of primary neurons is inhibited when all cells are artificially caused to express *X-Dl1* [21]. The scattered distribution of *Xcoe2*-induced neurons indicates that *Xcoe2* neural function is regulated by lateral inhibition. This regulation occurs in the epidermis as a result of ectopic activation of *X-Dl1* expression by injected *Xcoe2*. The sensitivity of *Xcoe2* neural function to lateral inhibition was confirmed by co-injecting into embryos *Xcoe2* mRNA and an mRNA

**Figure 5**




Induction of ectopic neurogenesis by injection of *Xcoe2* mRNA. Ectopic neurogenesis was visualised by whole-mount *in situ* hybridisation using either (a,c) *XNeuroD* or (b) *X-ngnr-1* probes on embryos at (a,b) stage 15 and (c) stage 25 or (d,e) immunostaining stage 30 embryos with anti-N-CAM antibodies. Embryos were microinjected with *Xcoe2* (400 pg) and *lacZ* (50 pg) mRNAs on one side (inj), and the other side was used as a control. An enlargement of the box in (d) is shown in (e).

**Figure 6**




*Xcoe2* ectopically activates, and is regulated by, lateral inhibition. One cell of a *Xenopus* embryo at the two-cell or four-cell stage was injected with (a–d) *XCoe2* (400 pg) and *lacZ* (50 pg) mRNAs; a combination of (e) *XCoe2* (10 pg), *X-Dl1*$^{dn}$ (400 pg) and *lacZ* (50 pg) mRNAs; or (f) *X-Dl1*$^{dn}$ (400 pg) and *lacZ* (50 pg) mRNAs, as indicated. Injected embryos at (a) stage 12.5, (b,c) stage 17 or (d–f) stage 13.5–14 were hybridised with (a,b) *X-Dl1* probe, (c) a combination of *X-Dl1* and *N-tubulin* probes or (d–f) *N-tubulin* probe alone as indicated. (a–c) Lateral view, the injected side is shown. (d–f) Dorso-lateral view, the injected side is indicated (inj). Ectopic *X-Dl1* expression is fairly uniform at stage 12.5 on the injected side, inj (a), and concentrates in patches of cells at stage 17 (b). At stage 17 (c), ectopic *N-tubulin* expression (dark blue staining) occurs specifically at the periphery of *X-Dl1* positive patches (red staining).

encoding a dominant-negative form of *X-Dl1* (*X-Dl1*$^{stu}$, renamed *X-Dl1*$^{dn}$) [21,22]. Co-expression of *Xcoe2* and *X-Dl1*$^{stu}$ resulted in a dramatic increase in both the penetrance of ectopic neurogenesis (93% of embryos; $n = 46$) and the density of supernumerary neurons induced by *Xcoe2* in both the neural plate and the epidermis (compare Figure 6d and e), whereas injections of *X-Dl1*$^{dn}$ alone lead to supernumerary neuron formation only in the neural plate (Figure 6f) [21]. We conclude from these experiments that *Xcoe2* positively regulates the transcription of *XDl1*, and that its ability to drive neuronal differentiation is highly sensitive to lateral inhibition (Figure 7).

## Discussion

In this report, we have investigated the role of XCoe2, an HLH transcription factor of a new type, in the specification of primary neurons in the *Xenopus* embryo. Results from both gain-of-function and loss-of-function experiments lead us to propose that *Xcoe2* is required for the stable commitment of selected progenitor cells to a neural fate. XCoe2 acts downstream of *X-ngnr-1* to maintain both Delta–Notch signalling and high levels of *X-ngnr-1* expression in selected progenitor cells and is required for the activation of neural differentiation genes such as *XNeuroD*.

### XCoe2, an HLH factor in the transcriptional activation cascade controlling primary neurogenesis

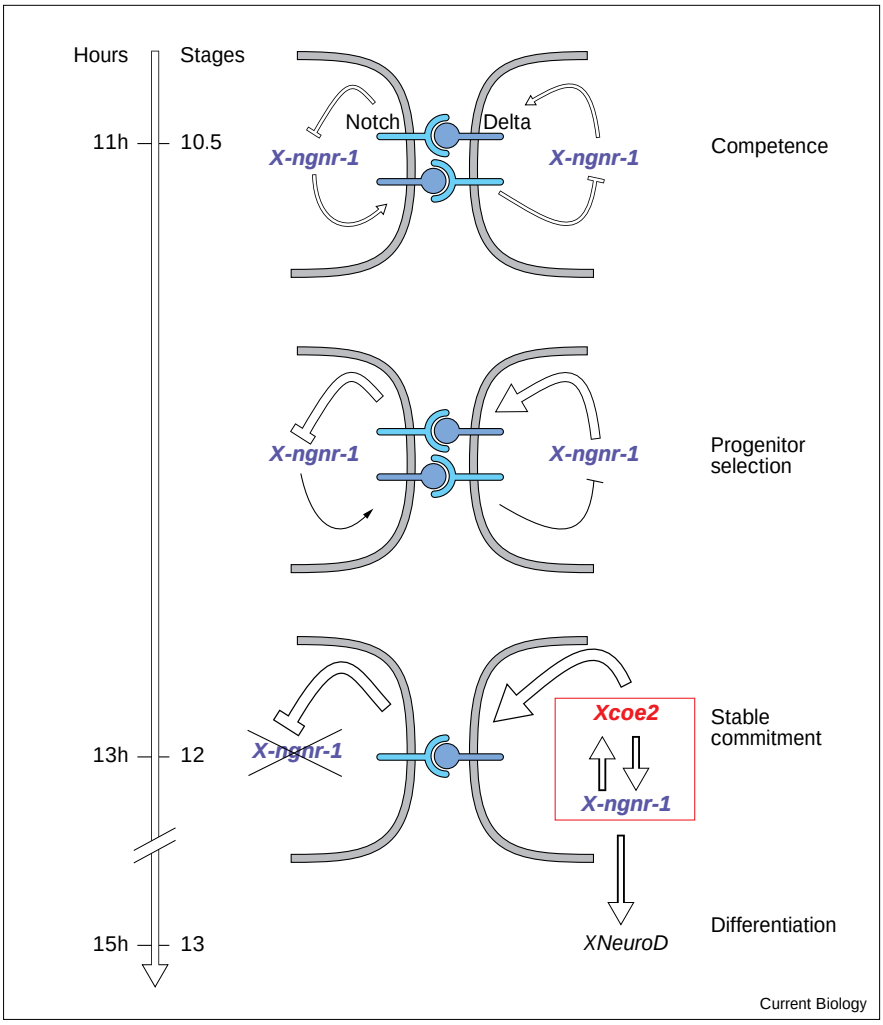
*Xcoe2* is transiently expressed in differentiating primary neurons and is both necessary for primary neurogenesis and able to convert epidermal cells into neurons. Temporally, *Xcoe2* is activated after *X-ngnr-1* and before *XNeuroD* in spatially overlapping patterns within the three prospective areas of primary neurogenesis. Gain-of-function experiments show that *X-ngnr-1* is able to activate the transcription of *Xcoe2*, and *Xcoe2* can in turn activate *X-Dl1* and *XNeuroD*, as well as *X-ngnr-1*, in a positive-feedback mechanism. Conversely, in the presence of a dominant-negative mutant form of XCoe2, the selected progenitor cells which express *Xcoe2* lose *X-ngnr-1* expression and do not commit to a neural fate. Together, these results suggest that *Xcoe2* plays a pivotal role during primary neurogenesis in enforcing *X-ngnr-1* proneural function and in promoting neuronal differentiation of committed cells by activating the expression of genes such as *XNeuroD* either directly or indirectly (perhaps through the maintenance of *X-ngnr-1* expression). Activation of *Xcoe2* occurs at stage 12, whereas *XNeuroD* transcripts accumulate from stage 13.5 ([9,10,23] and this report). The time window between activation of *Xcoe2* and *XNeuroD*, during which the vast majority of primary neuron progenitors are in the last G2 phase preceding their exit from the mitotic cycle [1], might allow specified neuronal progenitors to interact with spatio-temporally regulated signals in their microenvironment, and to adopt the fate they will express post-mitotically, in a cell-autonomous manner.

### From selection to differentiation of primary neural progenitors

Expression of the proneural *achaete-scute* complex (*AS-C*) genes in the *Drosophila* neurectoderm defines domains in which all cells are competent to become neuroblasts, as revealed in neurogenic mutants [24]. Only a selected number of these cells do, however, become irreversibly committed to a neural fate. Whereas the transcriptional feedback loop involving Notch–Delta signalling may not provide the main mechanism of neuroblast selection, it is required for the refinement/maintenance of this selection process so as to ensure the segregation of the correct number of neuroblasts at the right time and position [25]. This, together with experiments in *Xenopus* embryos and retinae and the embryonic chick retina, indicates that Delta–Notch signalling regulates the proportion of progenitors that become committed to the neuronal fate [22,25–27].

Whereas expression of the proneural-type gene *X-ngnr-1* makes a cell competent to become a neuronal progenitor, the factors that stabilise the competent state and control

**Figure 7**




Model for the transcriptional interactions controlling the specification of primary neurons in *Xenopus*. *X-ngnr-1, Xcoe2 and XNeuroD* are sequentially activated in primary neuron progenitors. Expression of *X-ngnr-1* renders cells competent to adopt a neural fate [10]. Expression of X-Ngnr-1 above a certain threshold leads to activation of *Xcoe2* in selected progenitor cells. XCoe2 activity is required for stable commitment of these cells to a neural fate via the maintenance of Delta–Notch signalling and a positive-feedback regulation of *X-ngnr-1* expression. XCoe2 is also required for the expression of differentiation genes such as *XNeuroD*. Whether this activation is direct or is through the maintenance of high levels of *X-ngnr-1* expression, or both, remains to be determined. The time window between activation of *Xcoe2* and *XNeuroD*, at stages 12 and 13.5, respectively, during which the majority of primary neurons are in the last G2 phase preceding their exit from the mitotic cycle [1], might allow neuron progenitors to respond to spatially and temporally regulated environmental cues.

the transition between competence and irreversible commitment to become a primary neuron remained to be defined [10]. That XCoe2 is such a factor is supported by several lines of evidence. First, Xcoe2 is required for the maintenance of *X-ngnr-1* expression in selected progenitor cells during their transition from selection to differentiation. Second, Xcoe2 is able to activate *X-Dl1* expression. Third, its ability to ectopically activate *X-ngnr-1* and *XNeuroD* in only scattered cells indicates that its neural-promoting function is highly sensitive to lateral inhibition. The difference between the patterns of *X-Dl1* and *X-ngnr-1* expression also indicates that *X-Dl1* activation is a primary response to *XCoe2* overexpression rather than a secondary response mediated by X-Ngnr-1. This genetic interaction between *Xcoe2* and *Delta–Notch* signalling was confirmed by the increased density of ectopic neurons induced by overexpressed *Xcoe2* in the presence of a dominant-negative form of *X-Dl1*, *X-Dl1*$^{dn}$, which renders cells 'deaf' to lateral inhibition [21,22].

To date, XCoe2 is the only transcription factor whose ability to induce ectopic neurons in the epidermis is highly sensitive to lateral inhibition. Overexpression of the bHLH protein XASH-3 (*Xenopus* AS-C homologue-3) also results in a scattered pattern of ectopic neurons [26], but in contrast to XCoe2, XASH-3 promotes neurogenesis only in the neural plate [28,29]. Because *X-ngnr-1* (or *Xcoe2*) and *XASH-3* are expressed in largely non-overlapping groups of cells in the neural plate ([10,28–30] and this report), it is possible that different pathways of neuronal specification operate during *Xenopus* early embryogenesis, involving different genes with similar proneural functions, as is the case in *Drosophila* for *AS-C* and *atonal*. X-Myt, a zinc-finger transcriptional activator, has been proposed to cooperate with the proneural bHLH proteins X-Ngnr-1 and XASH-3 to promote their proneural activity in selected progenitor cells, although probably not by directly interfering with the lateral inhibitory circuit [23]. Whether X-Myt plays a role in the positive-feedback regulation of *X-ngnr-1* remains to be examined.

### A family of HLH transcription factors involved in vertebrate neural development

*Xcoe2* was isolated on the basis of sequence homology to *Drosophila col* and rodent *EBF/Olf-1*. Using the same strategy, two new mouse genes have been isolated recently [15,17]: *EBF-2/OE3* and *EBF-3/OE2*, closely related to *EBF/Olf-1* (henceforth renamed *EBF-1/OE1*). The three mouse genes show largely overlapping expression patterns during embryonic development of the nervous system, in early post-mitotic cells in the spinal cord, midbrain and specific areas of the forebrain, which might explain why no obvious neuronal phenotype was identified in *EBF-1* knockout mice [31]. The transient expression of *EBF/OE* in cells withdrawing from the mitotic cycle in the spinal cord is reminiscent of that observed for *Xcoe2* during primary neurogenesis. XCoe2, the EBFs and Col thus define a family of transcription factors expressed during neuronal specification (this report and M.C., data not shown) [15,17] characterised by a new type of DNA-binding domain associated with an HLH motif. We refer to these proteins under the generic name of Coe transcription factors.

HLH motifs, which mediate homodimerisation and/or heterodimerisation, have so far been found in two types of transcription factor: bHLH proteins, all of which bind to similar DNA sequences collectively designated E boxes; and proteins lacking the basic region. This second group of proteins has been shown to antagonise the function of bHLH proteins by forming heterodimers that are unable to bind DNA. Although there is no evidence so far for heterodimer formation between Coe proteins and other types of HLH proteins, it has been established previously that EBF-1 binds to DNA as a dimer [12,14,32,33]. Furthermore, all three mouse Coe proteins are able to dimerise with themselves and with the other members of the family [17]. Recently, EBF-1 and the bHLH protein E47 have been shown to collaborate to induce expression of the early B-lymphocyte-specific gene λ5. In this case, the synergy between the two transcription factors relies upon the multiplicity of their binding sites in the λ5 promoter rather than synergistic protein–protein interactions [33]. Complex families of bHLH transcription factors related to the *Drosophila* proneural proteins AS-C or Atonal (the NeuroD, Neurogenin or MATH families) have been identified recently in the mouse and chicken [34–37]. Thus, there are already a large number of putative neuronal determination or differentiation factors with which Coe proteins potentially interact, in both the central and peripheral nervous systems. Like the EBFs [15,17], the several *neurogenin* genes in the mouse also form a family with partly overlapping expression patterns in the spinal cord, the brain, neural crest cells or the olfactory placodes, which might activate *NeuroD* in different sublineages [34,35]. It remains a challenging question whether interaction between different Coe proteins and bHLH proteins in the nervous system is limited to directional cascades of transcriptional activation or involves synergistic interactions on specific promoters to activate subsets of genes in a combinatorial fashion.

## Conclusions

This analysis reveals an important role for XCoe2, an HLH transcription factor (of the Coe family), in the specification of primary neurons in *Xenopus*. Both the temporal pattern of *Xcoe2* expression and the requirement for *Xcoe2* in order for selected progenitor cells to become committed to a neural fate define a new step in primary neurogenesis: a switch between neural competence (conferred by the proneural-like gene *X-ngnr-1*) and neuronal differentiation (Figure 7). Like *Xcoe2* and *zfcoe2* in the zebrafish (L.B-C., unpublished observations), *EBF/OE* genes in the mouse are transiently expressed in differentiating neurons in the CNS, in the zone extending from the midbrain to the spinal cord and in specific regions of the anterior brain [15]. These data are consistent with a conserved role for Coe transcription factors in the control of neuronal differentiation in the vertebrate CNS.

## Materials and methods

### *Isolation of cDNA*

Random-primed cDNA was prepared from embryonic stage 25 *Xenopus* RNA. Degenerate oligonucleotide primers were designed, based on conserved amino-acid sequences within the DNA-binding domain of the *Drosophila* Col and mouse EBF proteins [15]: 3′ degenerate primer 5′TT(A/G)TT(A/G)TGNAC(A/G)AACAT(A/G)TT(A/G)TC3′ corresponds to the peptide sequence DNMFVHNN (in single-letter amino-acid code) and 5′ degenerate primer 5′GCNCA(C/T)-TT(T/C)GA(A/G)AA(A/G)CA(A/G)CC3′ corresponds to the peptide sequence AHFEKQP. These primers were used for PCR amplification under conditions described by the manufacturer (Cetus). Amplification was for 30 cycles at 94°C for 1 min, 50°C for 2 min and 72°C for 3 min. PCR products of the appropriate size (560 bp) were purified by gel electrophoresis, subcloned and sequenced. The fragment corresponding to the most abundant class of PCR products was used as a probe to screen a stage 17 embryo cDNA library in λgt10. Six cDNAs that encode Col/EBF-related proteins were identified in a screen of $10^6$ colonies. The largest cDNA (2.2 kb), which contains a complete open reading frame, was subcloned in pBSKS, and its nucleotide sequence was determined using the Pharmacia sequencing kit. The GenBank Accession number for the *Xcoe2* sequence is AF041138.

### In situ *hybridisation, antibody staining and histology*

The 2.2 kb *Xcoe2* cDNA, cloned in pBSKS, was linearised with *Xba*I and transcribed with T3 RNA polymerase to generate a digoxigenin-labelled antisense RNA probe. The 5′ *Xcoe2* probe was generated using the 5′ *Eco*RI–*Afl*II *Xcoe2* cDNA fragment (686 bp) cloned in pBSKS. The *XNeuroD*, *X-Dl1* and *N-tubulin* probes were prepared as described [9,26,38]. Embryos were staged according to Nieuwkoop and Faber [39]. Whole-mount *in situ* hybridisation was performed using the procedure of Harland [40], with the following modifications: pre-blocking of the alkaline-phosphatase-conjugated digoxigenin antibody [41]; incubation of embryos for 1 h at room temperature in blocking solution (Boehringer Mannheim); and staining with BM purple (Boehringer Mannheim). Double-labelling whole-mount *in situ* hybridisation was performed as described [22], using digoxigenin-labelled *X-Dl1* and fluorescein-labelled *N-tubulin* RNA probes. Embryos were stained with anti-N-CAM antibody (1/20 dilution) as described [42], following *in situ* hybridisation or immunohistochemistry; some embryos

were cleared in 2:1 benzyl benzoat : benzyl alcohol. Sections (10 µm) were made from stained paraffin-embedded embryos.

### *RNA synthesis and injection*

The entire *Xcoe2* cDNA was cloned into the *Eco*RI site of the RN3 vector [43]. To synthesise RNAs coding for a truncated XCoe2 protein lacking part of the DNA-binding domain (XCoe2$^{\Delta DBD}$), the *Pst*I–*Xho*I 3′ fragment of *Xcoe2* (corresponding to amino acids 303–589) was cloned in-frame into the *Stu*I site of the plasmid pCS2+MT [28]. Capped *Xcoe2* and *Xcoe2*$^{\Delta DBD}$ mRNAs were transcribed using the T3 and SP6 Cap-Scribe, respectively (Boehringer Mannheim). *X-ngnr-1* [10], *X-Dl1*$^{dn}$ and *NLSlacZ* [21] RNAs were prepared as originally described, and were injected at a concentration of 2–100 pg/nl, in a volume of 5 nl, into a single blastomere of two- to eight-cell embryos. In most experiments, *lacZ* mRNA was co-injected as a marker; injection of *lacZ* mRNA alone was used as a control. Histochemical staining for β galactosidase was used to visualise the distribution of injected mRNAs. Embryos were collected at either the neural plate stage (stage 12.5–15) or tailbud stage (stage 25–28) and subjected to *in situ* hybridisation as described above.

## Acknowledgements

We are grateful to Marc Haenlin, Fabienne Pituello, Julian Smith and Cathy Soula for comments on the manuscript and Françoise Foulquier and Karine Rizzoti for help with embryo injections and *in situ* hybridisation. We also thank David Anderson, Chris Kintner, David Ish-Horowicz, Jacqueline Lee and Tomas Pieler for DNA constructs, and Nancy Papalopulu for the neurula stage cDNA library. This study was supported by the Centre National de la Recherche Scientifique and grants from the Ministère de la Recherche et de l'Enseignement Supérieur (ACC SV4), the Association pour la Recherche sur le Cancer and the Human Science Frontier Organisation. L.D. was supported by fellowships from MESR and Fondation pour la Recherche Médicale, and L.B-C. by an EMBO long-term fellowship.

## References

1. Hartenstein V: Early neurogenesis in ***Xenopus***: the spatio-temporal pattern of proliferation and cell lineages in the embryonic spinal cord. *Neuron* 1989, 3:399-411.
2. Koplan R, Turner D: The Notch pathway: democracy and aristocracy in the selection of cell fate. *Curr Opin Neurobiol* 1996, 6:594-601.
3. Lewis J: Neurogenic genes and vertebrate neurogenesis. *Curr Opin Neurobiol* 1996, 6:3-10.
4. Artavanis-Tsakonas S, Matsuno K, Fortini ME: Notch signalling. *Science* 1995, 268:225-232.
5. Heitzler P, Simpson P: The choice of cell fate in the epidermis of ***Drosophila***. *Cell* 1991, 64:1083-1092.
6. Skeath JB, Carroll SB: Regulation of proneural gene expression and cell fate during neuroblast segregation in the ***Drosophila*** embryo. *Development* 1992, 114:939-946.
7. Kunisch M, Haenlin M, Campos-Ortega JA: Lateral inhibition mediated by the neurogenic gene Delta is enhanced by proneural genes. *Proc Natl Acad Sci USA* 1994, 91:10139-10143.
8. Heitzler P, Bourouis M, Ruel L, Carteret C, Simpson P: Genes of the ***enhancer of split*** and ***achaete-scute*** complexes are required for a regulatory loop between Notch and Delta during lateral signaling in ***Drosophila***. *Development* 1996, 122:161-171.
9. Lee JE, Hollenberg SM, Snider L, Turner DL, Lipnick N, Weintraub H: Conversion of ***Xenopus*** ectoderm into neurons by neuroD, a basic helix-loop-helix protein. *Science* 1995, 268:836-844.
10. Ma Q, Kintner C, Anderson DJ: Identification of neurogenin, a vertebrate neuronal differentiation gene. *Cell* 1996, 87:43-52.
11. Crozatier M, Valle D, Dubois L, Ibnsouda S, Vincent A: ***collier***, a novel regulator of ***Drosophila*** head development, is expressed in a single mitotic domain. *Curr Biol* 1996, 6:707-718.
12. Hagman J, Belanger C, Travis A, Turck CN, Grosschedl R: Cloning and functional characterization of Early B-cell factor, a regulator of lymphocyte-specific gene expression. *Genes Dev* 1993, 7:760-773.
13. Wang MM, Reed RR: Molecular cloning of the olfactory neural transcription factor Olf-1 by genetic selection in yeast. *Nature* 1993, 364:121-126.
14. Hagman J, Gutch MJ, Lin H, Grosschedl R: EBF contains a novel zinc coordination motif and multiple dimerisation and transcriptional activation domains. *EMBO J* 1995, 14:2907-2916.
15. Garel S, Marin F, Mattéi MG, Vesque C, Vincent A, Charnay P: A family of EBF/OLF-1 related genes potentially involved in neuronal differenciation and regional specification in the CNS. *Dev Dyn* 1997, 210:191-205.
16. Malgaretti M., Pozzoli O, Bosetti A, Corradi A, Ciarmatori S, Panigada M, *et al*.: Mmot1, a new helix-loop-helix transcription factor gene displaying a sharp expression boundary in the embryonic mouse brain. *J Biol Chem* 1997, 272:17632-17639.
17. Wang SS, Tsai RYL, Reed RR: The characterisation of the Olf-1/EBF-like HLH transcription factor family: implications in olfactory gene regulation and neuronal development. *J Neurosci* 1997, 17:4159-4169.
18. Kintner CR, Melton DA: Expression of ***Xenopus*** N-CAM RNA in ectoderm is an early response to neural induction. *Development* 1987, 99:311-325.
19. Brenner S: The genetics of ***Caenorhabditis elegans***. *Genetics* 1974, 77:71-94.
20. Sadaghiani B, Thiebaud T: Neural crest development in the ***Xenopus laevis*** embryo, studied by interspecific transplantation experiments and scanning electron microscopy. *Dev Biol* 1987, 124:91-110.
21. Chitnis A, Henrique D, Lewis J, Ish-Horowicz D, Kintner C: Primary neurogenesis in ***Xenopus*** embryos is regulated by a homologue of the ***Drosophila*** neurogenic gene Delta. *Nature* 1995, 375:761-766.
22. Henrique D, Hirsinger E, Adam J, Le Roux I, Pourquié O, Ish-Horowicz D, Lewis J : Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina. *Curr Biol* 1997, 7:661-670.
23. Bellefroid E, Bourguignon C, Hollemann T, Ma Q, Anderson, Kintner C, Pieler T: X-Myt1, a ***Xenopus*** C2HC-type zinc finger protein with a regulatory function in neuronal differenciation. *Cell* 1996, 87:1-20.
24. Ghysen A, Dambly-Chaudière C, Jan LY, Jan YN: Cell interactions and gene interactions in peripheral neurogenesis. *Genes Dev* 1993, 7:723-733.
25. Seugnet L, Simpson P, Haenlin M: Transcriptional regulation of Notch and Delta: requirement for neuroblast segregation in ***Drosophila***. *Development* 1997, 124:2015-2025.
26. Chitnis A, Kintner C: Sensitivity of proneural genes to lateral inhibition affects the pattern of primary neurons in ***Xenopus*** embryos. *Development* 1996, 122:2295-2301.
27. Dorsky RI, Chang W.S, Rapaport DH, Harris WA: Regulation of neuronal diversity in the ***Xenopus*** retina by Delta signalling. *Nature* 1997, 385:67-70.
28. Turner DL, Weintraub H: Expression of achaete-scute homologue 3 in ***Xenopus*** embryos converts ectodermal cells to a neural fate. *Genes Dev* 1994, 8:1434-1447.
29. Ferreiro B, Kintner C, Zimmermann K, Anderson D, Harris WA: XASH genes promote neurogenesis in ***Xenopus*** embryos. *Development* 1994, 120:3649-3655.
30. Zimmerman K, Shih J, Bars A, Collazo A, Anderson D: XASH-3, a novel achaete-scute homologue, provides an early marker of planar neural induction and position along the mediolateral axis of the neural plate. *Development* 1993, 119:221-232.
31. Lin H, Grosschedl R: Failure of B-cell differentiation in mice lacking the transcription factor EBF. *Nature* 1995, 376:263-267.
32. Tsai RYL, Reed R: Cloning and functional characterization of Roaz, a zinc finger protein that interacts with O/E1 to regulate gene expression: implications for olfactory neuronal development. *J Neurosci* 1997, 11:4159-4169.
33. Sigvardsson M, O'Riordan M, Grosschedl R: EBF and E47 collaborate to induce expression of the endogenous immunoglobulin surrrogate light chain genes. *Immunity* 1997, 7:25-36.
34. Sommer L, Ma Q, Anderson D: ***neurogenins***, a novel family of ***atonal***-related bHLH transcription factors, are putative mammalian neuronal determination genes that reveal cell heterogeneity in the developing CNS and PNS. *Mol Cell Neurosci* 1996, 8:221-241.
35. Cau, E, Gradwohl G, Fode C, Guillemot F: Mash1 activates a cascade of bHLH regulators in olfactory neuron progenitors. *Development* 1997, 124:1611-1621.

36. Lee J: Basic helix-loop-helix genes in neural development. *Curr Opin Neurobiol* 1997, 7:13-20.
37. Roztocil T, Matter-Sadzinski L, Alliod C, Ballivet M, Matter JM : NeuroM, a neural helix-loop-helix transcription factor, defines a new transition stage in neurogenesis. *Development* 1997, 124:3263-3272.
38. Oschwald R, Richter K, Grunz H: Localization of a nervous system-specific class II beta-tubulin gene in ***Xenopus laevis*** embryos by whole-mount ***in situ*** hybridisation. *Int J Dev Biol* 1991, 35:399-405.
39. Nieuwkoop P, Faber J: *Normal Table of* Xenopus laevis, Amsterdam: North-Holland; 1967.
40. Harland RM: ***In situ*** hybridisation: an improved whole-mount method for ***Xenopus*** embryos. *Methods Cell Biol* 1991, 36:685-695.
41. Islam N, Moss T: Enzymatic removal of vitelline membrane and other protocol modifications for whole mount ***in situ*** hybridisation of ***Xenopus*** embryos. *Trends Genet* 1996, 12:459.
42. Brivanlou AH, Harland RM: Expression of an engrailed-related protein is induced in the anterior neural ectoderm of early ***Xenopus*** embryos. *Development* 1989, 106:611-617.
43. Lemaire P, Garret N, Gurdon JB: Expression cloning of Siamois, a ***Xenopus*** homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. *Cell* 1995, 81:85-94.

**Because *Current Biology* operates a 'Continuous Publication System' for Research Papers, this paper has been published on the internet before being printed. The paper can be accessed from http://biomednet.com/cbiology/cub – for further information, see the explanation on the contents page.**